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A Pou5f1/Oct4 dependent Klf2a, Klf2b, and Klf17 regulatory sub-network contributes to EVL and ectoderm development during zebrafish embryogenesis

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ABSTRACT

In mammalian ES cells, the transcription factors Klf4 and Klf2 contribute to maintenance of pluripotency and self-renewal and are regulated by Pou5f1/Oct4. In the early zebrafish embryo Pou5f1/Oct4 is necessary for expression of three Klf2/4 family members, *klf2a*, *klf2b* and *klf17* (previously *klf4b*), similar to the regulation reported for mammalian ES cells. In this study, we analyzed blastula and gastrula stage Klf regulatory networks and their influence on zebrafish embryonic patterning. We show that Pou5f1 acts in combination with region-specific factors to activate *klf2a*, *klf2b*, and *klf17* in the superficial cell layer of the embryo. In addition, Pou5f1 acts together with the BMP signaling pathway to activate and maintain expression of *klf2a* and *klf2b* in a ventral ectodermal domain. We used microarray expression profiles of *klf2a*, *klf2b* and *klf17* knockdown and overexpression embryos to identify Klf target genes, which reveals that Klfs participate in specification of the extraembryonic enveloping layer (EVL). We discuss mechanistic implications of simultaneous activation of transcriptional targets by ubiquitous, like Pou5f1, and region-specific inducers, emerging as a common regulatory motif in early development.

Introduction

The gene regulatory network (GRN) maintaining pluripotency in mammalian ES and iPS cells is centered on the transcription factors Pou5f1 (Oct4) and Sox2, and has been intensively studied because of the high potential of ES and iPS cells for human medicine (Young, 2011). Much less is known about the roles of Pou5f1 and Sox2 GRNs in the developing embryo. Previously we and others demonstrated the evolutionary similarities of the target gene sets of Pou5f1 and SoxB1 transcription factors in zebrafish and of Oct4 and Sox2 in mammals (Okuda et al., 2010; Onichtchouk et al., 2010). Comparative studies of conserved downstream components of Pou5f1 regulatory networks in vertebrates are instrumental to uncover their shared and diverse features, and to understand their structure, evolution, and developmental functions. Klf2 and Klf4 factors recently received much attention due to their roles in somatic reprogramming (Takahashi and Yamanaka, 2006; Hall et al., 2009) and embryonic stem (ES) cell self-renewal in mammals (Li et al., 2005; Ivanova et al., 2006; Bruce et al., 2007; Jiang et al., 2008; Wei et al., 2009). In this paper, we focus on zebrafish members of the Klf family, direct transcriptional targets of the Pou5f1 GRN in both zebrafish embryos and ES cells.

The family of Krüppel-like factors (Klfs) is a group of evolutionary conserved zinc finger transcription factors with strong homology to the *Drosophila melanogaster* Krüppel protein (Turner and Crossley, 1999). Klfs are characterized by three zinc finger motifs at their C-terminal end, which mediate the DNA binding to GC-rich elements, like the CACCC-box (Miller and Bieker, 1993; Bieker and Southwood, 1995; Shields and Yang, 1998; Yet et al., 1998; Turner and Crossley,

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1999). The N-termini of Klf proteins are highly variable and mediate transcriptional activation and/or repression depending on the recruitment of co-factors (Bieker and Southwood, 1995; Yet et al., 1998; Chen and Bieker, 2001).

Four Klfs of the Klf2/4 subfamily have been reported in zebrafish (Kawahara and Dawid, 2000; Oates et al., 2001; Li et al., 2011). Zebrafish Klf2a and Klf2b were reported as co-orthologs of mammalian KLF2 (Oates et al., 2001). Two Klf genes, *klf4b* (former *biklf*; Kawahara and Dawid, 2000) and *klf4a* (Li et al., 2011), were shown to be related to the mammalian *Klf4* gene (Oates et al., 2001; Li et al., 2011). The phylogenetic position of zebrafish *klf4b* is not completely clear, as it was considered more close to KLF2 in a recent publication (Li et al., 2011) To address this question, we studied synteny relationships of *klf2a*, *klf2b*, *klf4a* and *klf4b/biklf* in 8 vertebrate species, including human, mouse, chick, lizard, *Xenopus* and three fish species. Surprisingly, *klf4b/biklf* was syntenic neither to *klf4* or *klf2*, but to *klf17*, also called *neptune* in Xenopus (Fig S1). As judged by protein alignment of Klf proteins from seven vertebrates (Fig S2), Klf17 proteins form a distinct branch, related to Klf2 and Klf4 (Fig. S2A). Mammalian Klf17 proteins strongly diverged from non-mammalian orthologs and from each other, even within the DNA-binding domain (Fig. S2B), indicating the rapid evolution of Klf17 proteins specifically in mammals. *klf4a* is syntenic to *Klf4. klf2a* and *klf2b* represent a fish-specific duplication of *Klf2*, as previously suggested (Figs. S1, S2; Oates et al., 2001).

klf4a in zebrafish has been reported to be maternally expressed (Li et al., 2011). Morpholino-based knockdown of Klf4a has revealed no early phenotype, but a later role in regulating intestinal proliferation and differentiation of intestinal epithelia (Li et al., 2011), which is similar to the role of mammalian Klf4 (Garrett-Sinha et al., 1996; Shields et al., 1996; Jenkins et al., 1998; Zhang et al., 2000; Katz et al., 2002, 2005). Klf4 controls the differentiation of specific epithelial tissues by activating tissue specific keratin genes (e.g. *Keratin 4*; Jenkins et al., 1998; Brembeck and Rustgi, 2000; Chen et al., 2003).

Two paralogous zebrafish genes, *klf2a* and *klf2b*, are activated zygotically, expressed in the ventral ectoderm at gastrula stages and later on in the forming blood vessels (*klf2a*) or in the epidermis (*klf2b*) (Oates et al., 2001). Klf2a is involved in cardiac valve development and required for establishing the blood flow in zebrafish (Vermot et al., 2009; Just et al., 2011; Wang et al., 2011) during organogenesis, a role which has also been described for Klf2 in mice (Kuo et al., 1997; Chiplunkar et al., 2013).

klf17/klf4b/biklf is activated zygotically (Kawahara and Dawid, 2000; Onichtchouk et al., 2010) and is strongly expressed in the involuting axial mesoderm and polster during gastrulation (Kawahara and Dawid, 2000; Oates et al., 2001; Gardiner et al., 2005). Later in development Klf17 is expressed in the hatching gland, lateral line system and intermediate cell mass (Gardiner et al., 2007). During organogenesis, Klf17 plays a crucial role in hatching gland formation and primitive erythropoiesis in zebrafish (Gardiner et al., 2005, 2007), similar to the Xenopus ortholog Klf17/Neptune (Huber et al., 2001; Kurauchi et al., 2010). Expression of the chick Klf17 ortholog in blood islands also suggests a function in blood development (Antin et al., 2010). While the roles Klf17 plays during mammalian development still await investigation, Klf17 was identified as a negative regulator of epithelial–mesenchymal transition and metastasis in breast cancer, based on the experiments with cell lines (Gumireddy et al., 2009; Iwanicki and Brugge, 2009). During mouse embryogenesis, *Klf17* is strongly expressed only in testis and ovary (Yan et al., 2002).

While the roles of Klf2/4 family genes during organogenesis are well studied and conserved among vertebrates, their expression at blastula and gastrula stages is more variable and less is known about their functions during these early stages, which are vastly different between vertebrates. The first three hours of zebrafish development are driven by maternally provided gene products. The zygotic genome gets activated at around 3 h post fertilization (hpf) during midblastula transition (MBT). Approximately 1 h after MBT, the first morphologically differentiated cells in the embryo become visible: superficial blastomeres separate and form the extraembryonic enveloping layer (EVL), while the deep layer (DEL) cells give rise to all embryonic tissues (Kimmel et al., 1990; Sagerstrom et al., 2005). The EVL functions as a protection barrier between DEL cells and the surrounding environment (Kimmel et al., 1990, 1995; Zalik et al., 1999; Sagerstrom et al., 2005; Kiener et al., 2008). Previous studies reported that the EVL also plays an important role in gastrulation by taking an active part in epiboly (Kane et al., 1992; Zalik et al., 1999; Shimizu et al., 2005; Lachnit et al., 2008). Several maternal factors were shown to be crucial for EVL differentiation, including Irf6 (Fukazawa et al., 2010; de la Garza et al., 2013), IKK1 (Sabel et al., 2009), Pou5f1 (Lachnit et al., 2008), FoxH1 (Pei et al., 2007), and EpCAM (Slanchev et al., 2009). However, our understanding of the molecular interactions and the mechanism behind EVL differentiation is still incomplete.

Previous work of our lab (Onichtchouk et al., 2010) demonstrated that Pou5f1 regulates expression of *klf17*, *klf2a* and *klf2b* at stages when most zebrafish embryonic deep cells are pluripotent (Ho and Kimmel, 1993). In this study, we describe the earliest expression domains of *klf17*, *klf2a* and *klf2b* in detail and elucidate the role of Pou5f1 in controlling their expression. We demonstrate that Pou5f1 activates *klf17*, *klf2a* and *klf2b* in the surface cells of the blastula (EVL), acting together with an EVL-specific inducer, potentially Irf6. We show that within the EVL domain, Klf17, Klf2a and Klf2b are redundantly involved in differentiation by activating EVL specific differentiation genes. Pou5f1 also activates *klf2a* and *klf2b* in their ectodermal domain, acting synergistically with the BMP pathway. We used microarray expression profiles of *klf2a*, *klf2b* and *klf17* knockdown and overexpression embryos to identify Klf target genes. Judged from these data, Pou5f1-dependent Klfs may serve as redundant repressors of alternative cell fates within the ectodermal domain. Thus, in both EVL and ectodermal domains, closely related Klf factors are activated by synergistic cues, which include non-region-specific (Pou5f1), and region-specific (EVL inducer, BMP pathway) signals. This synergistic regulation of several closely related TFs may have facilitated rapid gain and loss of expression domains, contributing to the rapid evolution of early post-MBT gene regulatory networks.

Results

After MBT klf17 is specifically activated in the EVL by Pou5f1

It was previously reported that *klf17* is strongly activated in zebrafish after MBT (Fig. S3A; Kawahara and Dawid, 2000; Onichtchouk et al., 2010), but the early localization and regulation of its expression was so far unknown. Therefore, we examined the *klf17* expression pattern by whole-mount *in-situ* hybridization (WISH) in wild-type (WT), maternal (M*spg*), and maternal-zygotic Pou5f1 mutant (MZ*spg*) embryos at five different embryonic stages, starting at 1000-cell stage (MBT) and up to 75%-epiboly stage (Fig. 1). In WT, *klf17* expression was first detectable in EVL cells at sphere stage (Fig. 1B), and continues to be highly expressed in the EVL (Fig. 1C,D) until expression fades at the end of gastrulation (Fig. 1E). At the onset of emboly in gastrulation a second *klf17* expression domain appears in the involuting axial mesoderm (Fig. 1D,E white arrow; Kawahara and Dawid, 2000). To determine whether the strong *klf17* WISH signal in the EVL may obscure additional *klf17* expression domains in underlying tissues, we examined sections of whole-mount stained embryos

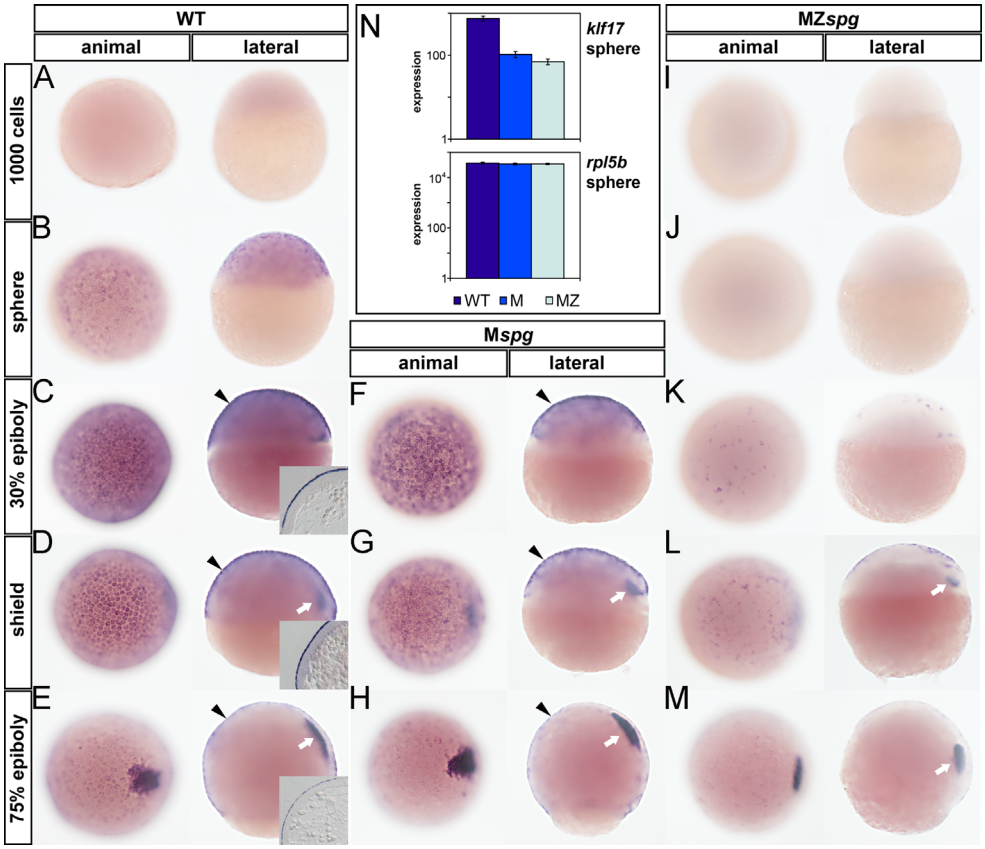

Fig. 1. *klf17* expression in the EVL shortly after MBT depends on Pou5f1. Analysis of *klf17* expression at embryonic stages from 1000 cells to 75% epiboly in WT (A–E), M*spg* (F–H) and MZ*spg* embryos (I–M). All embryos are shown in animal (left column) or lateral view (right column); shield and 75% epiboly stages are oriented with dorsal to the right. Insets show lateral regions of transversal sections of embryos. (N) Microarray data for *klf17* expression in WT, M*spg* and MZ*spg* embryos at sphere stage; *rpl5b* is not regulated by Pou5f1 and was used as normalization control.

(Fig. 1C–E; insets), but did not detect additional *klf17* expression in deep tissues.

In Pou5f1 deficient MZ*spg* mutants *klf17* expression was not detectable by WISH (Fig. 1J) and reduced in microarray data (Fig. 1N, microarray data from Onichtchouk et al., 2010) at sphere stage. During gastrulation *klf17* expression was strongly reduced in MZ*spg* mutants and could be detected only in very few EVL cells (Fig. 1K–M; Fig. S3A). In contrast, the second *klf17* expression domain in the involuting axial mesoderm remains unaltered in MZ*spg* embryos (Fig. 1L,M, white arrow) and therefore seems to be independent of Pou5f1 activity.

In M*spg* embryos, which express functional Pou5f1 starting from 1000 cell stage, expression of *klf17* is nearly as low as in MZ*spg* at sphere stage (Fig. 1N, microarray data from Onichtchouk et al., 2010). During gastrulation, *klf17* is expressed in EVL cells of M*spg* embryos at levels intermediate between those of MZ*spg* and WT (Fig. 1F–H). The *klf17* expression domain in the involuting axial mesoderm remains unaltered in M*spg* embryos (Fig. 1G,H, white arrow).

Our data demonstrate the existence of two distinct and differentially regulated *klf17* expression domains. First, *klf17* is activated by Pou5f1 in the extraembryonic EVL. In contrast, the second *klf17* domain in involuting axial mesoderm is Pou5f1-independent.

Two klf2b domains in EVL and ectoderm are differentially regulated by Pou5f1

Expression of *klf2a* and *klf2b* during late gastrulation was previously reported (Oates et al., 2001), but earlier expression was not characterized, and regulation is unknown. We examined expression patterns of *klf2a* and *klf2b* in WT, M*spg* and MZ*spg* embryos from 256-cell until late gastrula stages (Fig. 2 and S4). In WT embryos, weak diffuse expression of *klf2b* starts at sphere stage (Fig. S4E,F), but already at 30% epiboly *klf2b* becomes localized to the ventral part of prospective ectoderm and to some EVL cells (Fig. 2A, inset). At subsequent stages, expression of *klf2b* within the ventral ectoderm becomes stronger, while staining in the EVL fades to the end of gastrulation (Fig. 2B,C, insets). In MZ*spg* embryos, expression of *klf2b* was not detectable until 75% epiboly stage (Fig. 2G,H) and first appeared at 75% epiboly in the ventral ectodermal domain, which was significantly smaller compared to WT (Fig. 2I, compare to Fig. 2C). In M*spg* embryos, *klf2b* was present in both EVL and blastoderm. Compared to WT, expression of *klf2b* in EVL of M*spg* was stronger at 30% epiboly and shield stages (Fig. 2D,E, compare to Fig. 2A,B), but not at 75% epiboly (Fig. 2F, compare to Fig. 2C). This transient increase in *klf2b* expression was not seen in the deep cell domain (Fig. 2A–F), indicating that *klf2b* is differentially regulated in EVL and deep cells. Given that expression of transcriptional targets of Pou5f1 is delayed in M*spg* embryos (Onichtchouk et al., 2010), we hypothesized that upregulation of *klf2b* in M*spg* at 30% epiboly and shield may be due to the absence of a Pou5f1-dependent EVL-specific repressor of *klf2b* during these stages.

klf2a requires Pou5f1 for expression in the ectoderm

EVL-specific expression of *klf2a* could be detected already at sphere to 30% epiboly stage (Fig. S4M,N; Fig. 2J, inset) in WT embryos. At shield stage, EVL expression of *klf2a* faded (Fig. 2K) and could be detected only after prolonged staining (Fig. S4O,P).

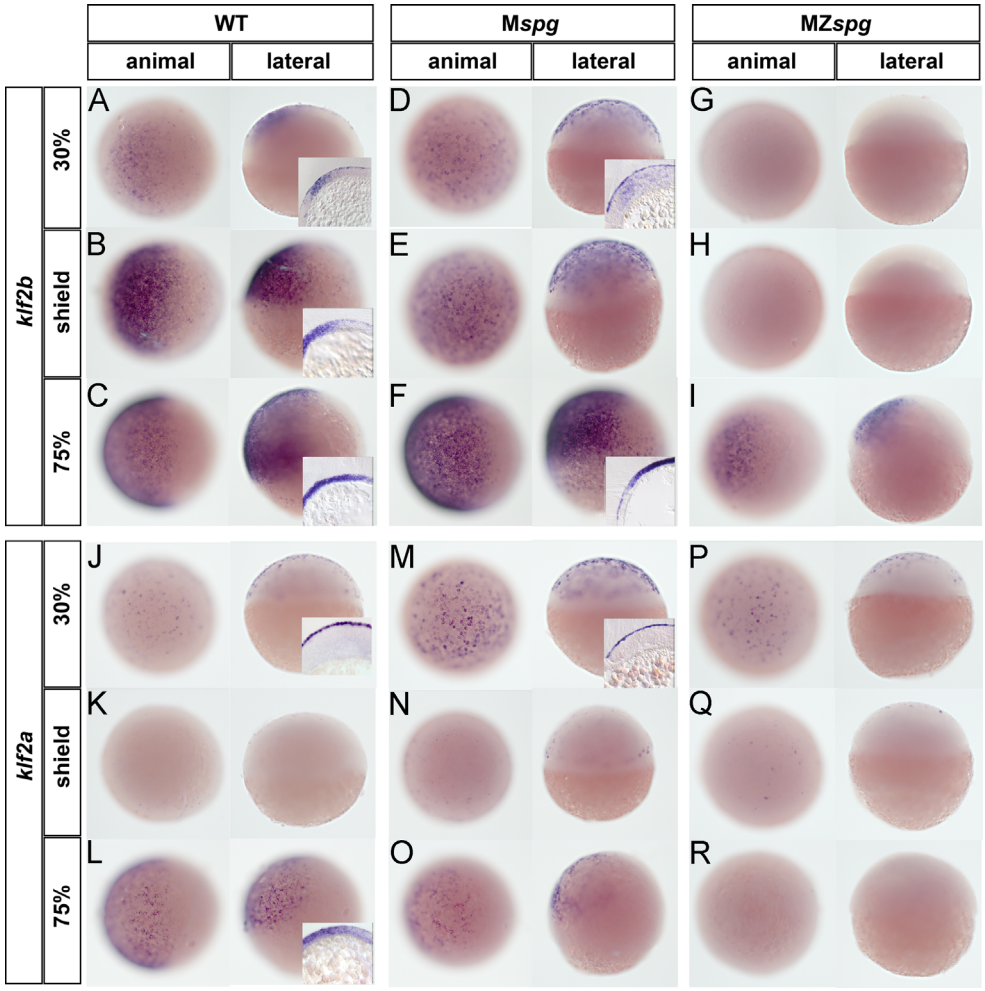

Fig. 2. *klf2a* and *b* are differentially regulated by Pou5f1. *klf2a* (A–I) and *klf2b* (J–R) expression analysis by WISH at three different gastrula stages, 30%-epiboly, shield and 75% epiboly. All embryos are shown in animal (left column) or lateral view (right column); shield and 75% epiboly stages are oriented with dorsal to the right. Inserts show lateral regions of transversal sections.

At 75% epiboly, *klf2a* expression appeared in the ventral ectoderm in WT (Fig. 2L). This ventral expression domain of *klf2a* is not detectable in MZ*spg* (Fig. 2R) and very weak in M*spg* (Fig. 2O). Therefore we concluded that Pou5f1 is necessary for expression of *klf2a* in the ventral ectodermal domain. Intensity of *klf2a* WISH staining in the EVL at 30% epiboly was similar in WT and MZ*spg* (Fig. 2J,P), but surprisingly higher in M*spg* mutant embryos (Fig. 2M). Higher levels of *klf2a* expression in M*spg* compared to MZ*spg* mutants suggest that zygotic Pou5f1 expression contributes to establishing the proper *klf2a* expression level in the EVL. This may potentially be caused by maternally present Pou5f1 at MBT inducing a hypothetical early zygotic repressor of *klf2a* expression, which may also reduce expression of *klf2a* in the EVL of WT embryos. In M*spg* embryos, the expression of this repressor may be delayed, as Pou5f1 is present only after MBT. In the EVL, Pou5f1 thus may provide both direct activating and indirect delayed repressing inputs to *klf2a* expression, as judged from differences of the expression patterns in WT, M*spg* and MZ*spg* embryos.

BMP signaling regulates Klf2b within its ectodermal domain

Dorso-ventral asymmetry of *klf2a* and *klf2b*, but not *klf17* expression, suggests that *klf2a* and *klf2b* may be activated by the BMP pathway, in addition to Pou5f1. To evaluate the contribution of BMP signaling to early expression of *klf2a* and *klf2b*, we analyzed the response of *klf2a* and *klf2b* at sphere and 30% epiboly to perturbations of the BMP pathway (Fig. 3). Overexpression of BMP2b resulted in robust upregulation of *klf2b* in the blastoderm (Fig. 3A,D and G,J), but resulted only in a very moderate activation of *klf2a* (Fig. 3M,P and S,W). Inhibition of BMP signaling by overexpression of *noggin1* (Furthauer et al., 1999), or repression of BMP2 transcription by overexpression of Fgf8a (Furthauer et al., 2004) resulted in reduction of *klf2b* expression (Fig. 3H,K, compare to G), but had no effect on *klf2a* (Fig. 3T,X, compare to S). Repression of dorsalizing FGF signaling by SU5402 increased the level of *klf2b* expression at sphere stage (Fig. 3B,E) and expanded the *klf2b* domain dorsally at 30% epiboly (Fig. 3I,L), but did not change *klf2a* levels or distribution pattern at any stage (Fig. 3N,Q and U,Y). We conclude that while BMP signaling is necessary for expression of the ectodermal domain of *klf2b* already at 30% epiboly, *klf2a* expression in the ectoderm becomes dependent on BMP at later stages. In MZ*spg* embryos, *bmp2* is initially suppressed by high levels of Fgf8 immediately after MBT and starts to be expressed with delay (Reim and Brand, 2006; Belting et al., 2011). We tested, if de-repression of BMP by SU5402 inhibition of early FGF signaling can induce expression of *klf2b* in MZ*spg* at sphere stage, as it does in WT. However, in MZ*spg* de-repression of BMP signaling did not induce *klf2b* (Fig. 3C,F), suggesting that both Pou5f1 and BMP inputs are simultaneously required for activation of *klf2b* expression in deep cells of the ectoderm.

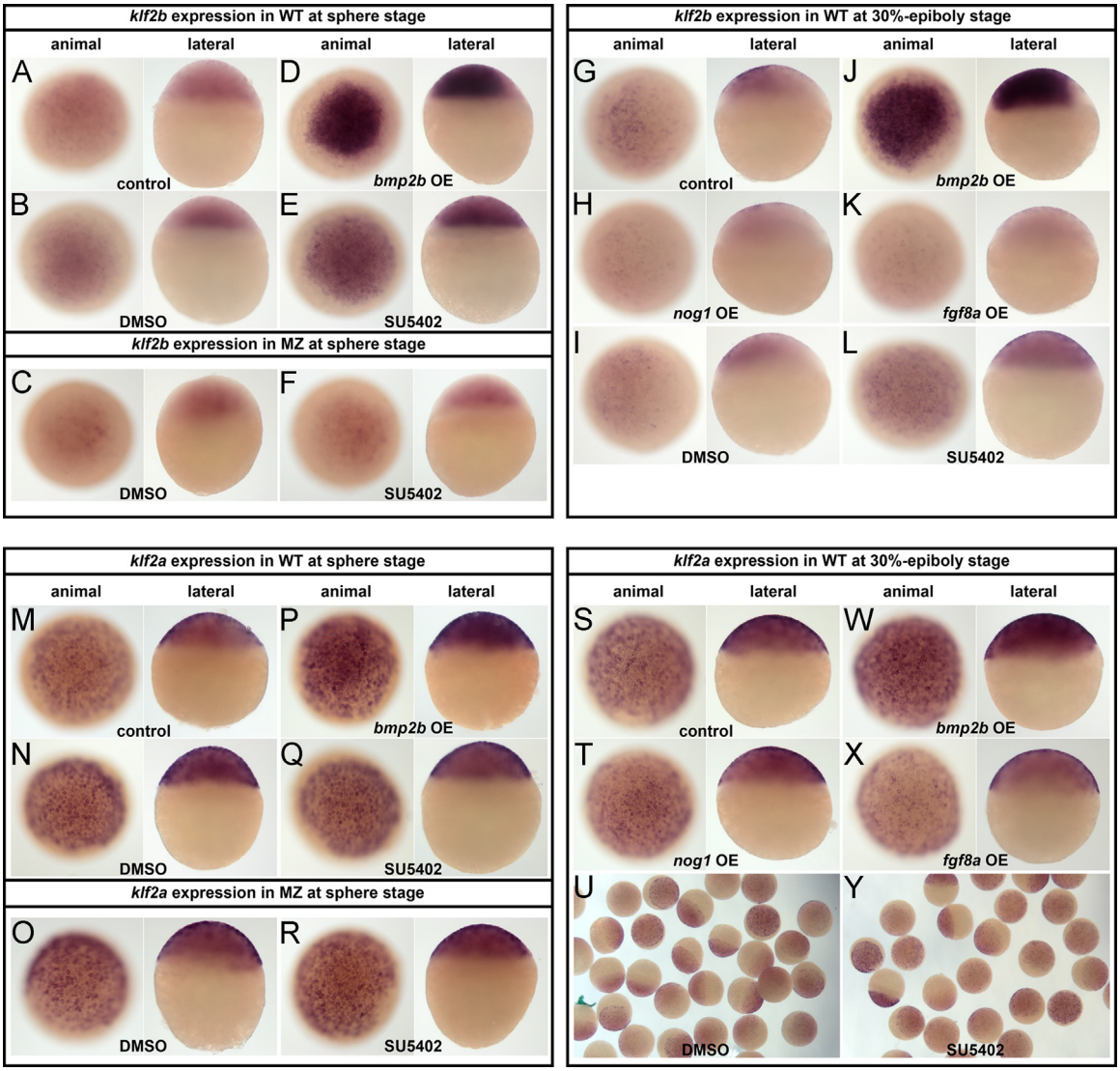

Fig. 3. Expression of *klf2a* and *b* is supported by Pou5f1 and BMP. Analysis *klf2b* (A–L, upper part) and *klf2a* expression (M–Y, lower part) at sphere (left panel) and 30% epiboly stages (right panel). Bmp2b overexpression strongly activates *klf2b* at sphere (D) and 30% epiboly stage (J). In addition, the inhibition of the FGF receptor by SU5402 leads to upregulation (E and L) and dorsal expansion of the *klf2b* expression domain (L), whereas Fgf8a overexpression results in a strong reduction of *klf2b* expression (K). In contrast, the expression of *klf2a* is only slightly enhanced by *bmp2b* overexpression (P and W) and a bit reduced by Fgf8a overexpression (X). No changes in expression levels were detectable after SU5402 treatment (F and Y). The inhibition of FGF signaling in MZ*spg* mutant embryos has no additional effect on the expression of both *klf2* genes (F and R). *nog1* overexpression has no effect on *klf2a* expression (T), while the expression levels of *klf2b* are reduced (H).

Overexpression of Klf2a, Klf2b and Klf17 directly induces EVL marker expression

To identify potential genomic targets of Klf proteins, we performed overexpression experiments by *klf2a*, *klf2b* or *klf17* mRNA injections in WT and MZ*spg* mutant embryos, and subsequently examined changes in expression profiles by microarray analysis (Fig. 4A; Materials and methods; Microarray data are submitted to GEO under accession number GSE45013). In Pou5f1 mutants, which have strongly reduced *klf* gene expression levels, the expression of Klf-dependent downstream targets should be rescued by Klf overexpression. In contrast, given that Klf overexpression from mRNA leads to ectopic expression, target genes with expression located outside of endogenous Klf expression domains may be repressed in the experiment. In parallel experiments, we identified potential direct Klf targets by performing *klf2a*, *klf2b* or *klf17* overexpression experiments in the presence of the translational inhibitor cycloheximide (CHX; Fig. 4A). In these experiments CHX is added at the 64 cell stage to suppress translation of zygotic transcripts after MBT. This allows translation of injected mRNAs, but inhibits the translation of zygotic transcripts and thus indirect effects on transcription caused by zygotic transcription factors (Leung et al., 2003). The differences in the expression profiles of *klf* injected plus CHX-treated compared to non-treated embryos and to non-injected control embryos should reveal genes directly regulated by each of the Klf factors.

To further select potential Klf direct targets, we took advantage of the facts that DNA-binding motifs for Klfs are known, and binding sites for Klfs are often located close to the basal promoter (Chen et al., 2008). Among the genes differentially expressed in Klf plus CHX overexpression experiments, we identified those genes with Klf binding motifs overrepresented in their upstream control regions. First, we detected shared motifs in a region within 200 bp upstream from transcription start site (TSS) using the MEME motif discovery tool (Bailey and Elkan, 1994), and subsequently compared the found motifs against the JASPAR database of known transcription factor binding motifs using the TOMTOM motif comparison tool (Gupta et al., 2007). Genes induced by Klf2a and

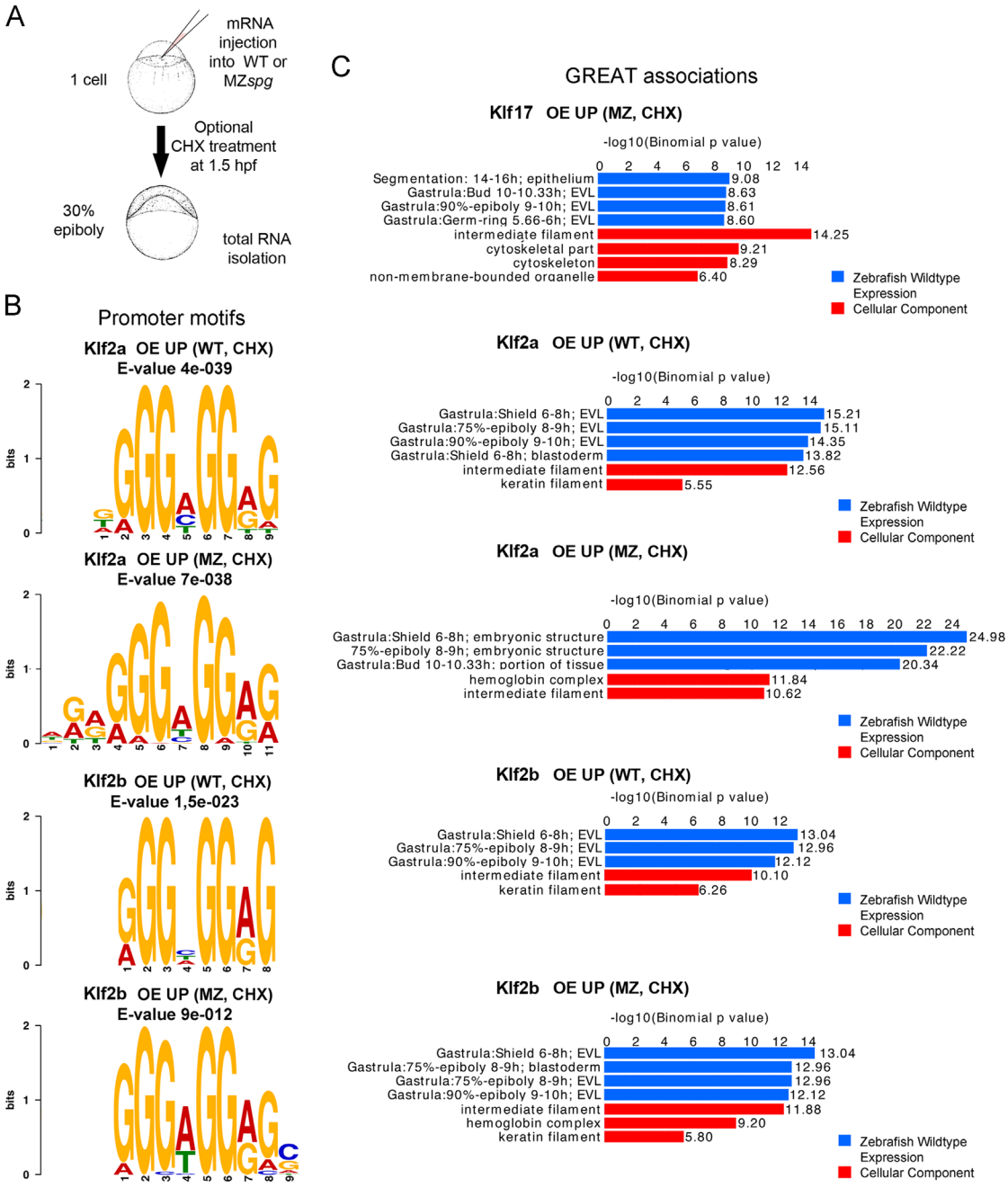

Fig. 4. Klf17, Klf2a and Klf2b directly activate EVL-specific differentiation genes. (A) Experimental setup to identify Klf2a, Klf2b and Klf17 targets by zebrafish transcriptome microarray analysis. Embryos were injected with mRNA encoding Klfs and treated with the inhibitor of translation cycloheximid (CHX) from the 64-cell stage on to suppress translation of zygotic transcripts and identify direct targets, while controls were untreated. (B) Position-Weight matrices (PWMs) for motifs overrepresented in the promoters of genes upregulated after Klf2a and Klf2b overexpression in embryos treated with CHX (KLF2a OE UP, KLF2b OE UP), in wildtype (WT) or MZ*spg* (MZ) embryos, match canonical SP1–Klf4 sites. The *E*-value represents the statistical significance of the motif. (C) Expression pattern and cellular component annotation correlation analysis of direct Klf17, Klf2a and Klf2b upregulated targets was performed using GREAT. The charts represent the top overrepresented categories belonging to zebrafish expression patterns (blue; structure and stage) and cellular components (red). The *x* axis values correspond to the binomial raw (uncorrected) *P*-values.

Klf2b in the presence of CHX were with high significance ($E < 10^{-11}$) enriched in motifs closely resembling known Klf4 binding matrices (Fig. 4B; Chen et al., 2008). This approach should select target genes in Klf2a and Klf2b overexpression experiments for which activation is caused by direct binding of Klfs to their cognate binding sites. Further, promoters of genes repressed by Klf17 overexpression in both WT and MZ*spg* in the absence of CHX were also strongly enriched in SP1–Klf4 like motifs (Fig. S5B). This suggests that Klf17 may also be able to act as a direct repressor at blastula stage. In contrast, promoters of genes repressed by Klf2b overexpression in WT and MZ*spg* embryos with or without CHX did not show enrichment for SP1–Klf4-like motifs, suggesting that direct repression is not a main mode of Klf2b action. Promoters of genes repressed by Klf2a were enriched for SP1–Klf4-like motifs only in Klf2a overexpression experiments in MZ*spg* embryos (Fig. S5A), suggesting that Klf2a "repressive" binding sites may be more accessible for Klf2a in MZ*spg* mutants. *klf17* is an example for a Klf2a target which is repressed only in MZ*spg* embryos (Fig. 6E).

We next used statistical correlation analysis to classify the identified potential Klf target genes by their expression patterns, biological process, or other GO annotations. The relationships between the genes identified as potential Klf targets and

annotated genes grouped by their expression or functional properties were analyzed using the GREAT web tool, linked to a variety of biologically relevant annotations from different sources (McLean et al., 2010). The groups of genes induced by Klf17, Klf2a, or Klf2b when post-MBT translation was inhibited by CHX were significantly associated spatially with EVL and blastoderm-expressed genes, and functionally with intermediate filament genes (Fig. 4C). Interestingly, in MZ*spg*, but not in WT embryos, Klf2a and Klf2b in addition to EVL markers also induced hemoglobin cluster genes, which are normally activated by Klf17 at later developmental stages. The correlation analysis of expression, biological process GO and pathway annotations showed that the genes downregulated by Klf17, but not by Klf2a or Klf2b overexpression, are associated with early gene transcription in different germ layers, developmental processes, and main embryonic pathways (Fig. S5B). Taken together, overexpression results suggest that all three Klfs studied here can directly activate genes involved in EVL development and differentiation.

Klf17 activates EVL target genes while Klf2b represses marginal region targets

To address potential functions of Klf17, Klf2a, and Klf2b in early embryogenesis we injected Klf17 translation blocking morpholinos (ATG-MO; (Kawahara and Dawid, 2001)), Klf2a ATG-MO and Klf2b ATG-MO (Materials and methods, Fig. S6) individually or in combinations into one cell stage embryos. Klf2a, 2b and 17 could be removed without compromising early embryo viability, as single, double, or triple gene knockdowns did not affect survival of embryos up to 24 hpf compared to control injections under standard laboratory conditions (data not shown). Robust developmental processes are often regulated by multiple TFs of different families (reviewed in: Macneil and Walhout, 2011), where the contributions of individual regulatory TFs can only be unmasked upon their removal under specific stress conditions or upon removal of all regulatory TFs. We next examined changes in gene expression levels in Klf morphants using mRNA microarrays to detect changes in gene expression levels.

GREAT correlation analysis reveals that transcripts reduced in Klf17 morphants are strongly enriched for genes expressed in the EVL or blastoderm at gastrula stages (Fig. 5A). These data suggest Klf17 to activate transcription in EVL development, which is consistent with the fact that *klf17* is most strongly expressed in EVL compared to *klf2a* and *klf2b*. A search for common motifs occurring in the promoters of genes downregulated in Klf17 KD reveals a canonical SP1–Klf4 site with high statistical significance (Fig. 5B). Promoters of genes downregulated upon *klf2a* knockdown were also significantly enriched in SP1–Klf4-type sites (Fig. 5C). Interpretation of overexpression and knockdown data suggests that direct binding of Klf17 to the upstream regulatory regions of its EVL target genes significantly contributes to their activation, while contribution of Klf2a and Klf2b to the regulation of EVL target genes is smaller, in accordance to their weaker expression in EVL.

Genes upregulated in the Klf2b KD experiment correlated with genes expressed in the mesodermal derivatives at gastrula stage, and at later stages with mesenchymal genes (Fig. 5D). Fig. 5E shows data for seven genes with known expression in the

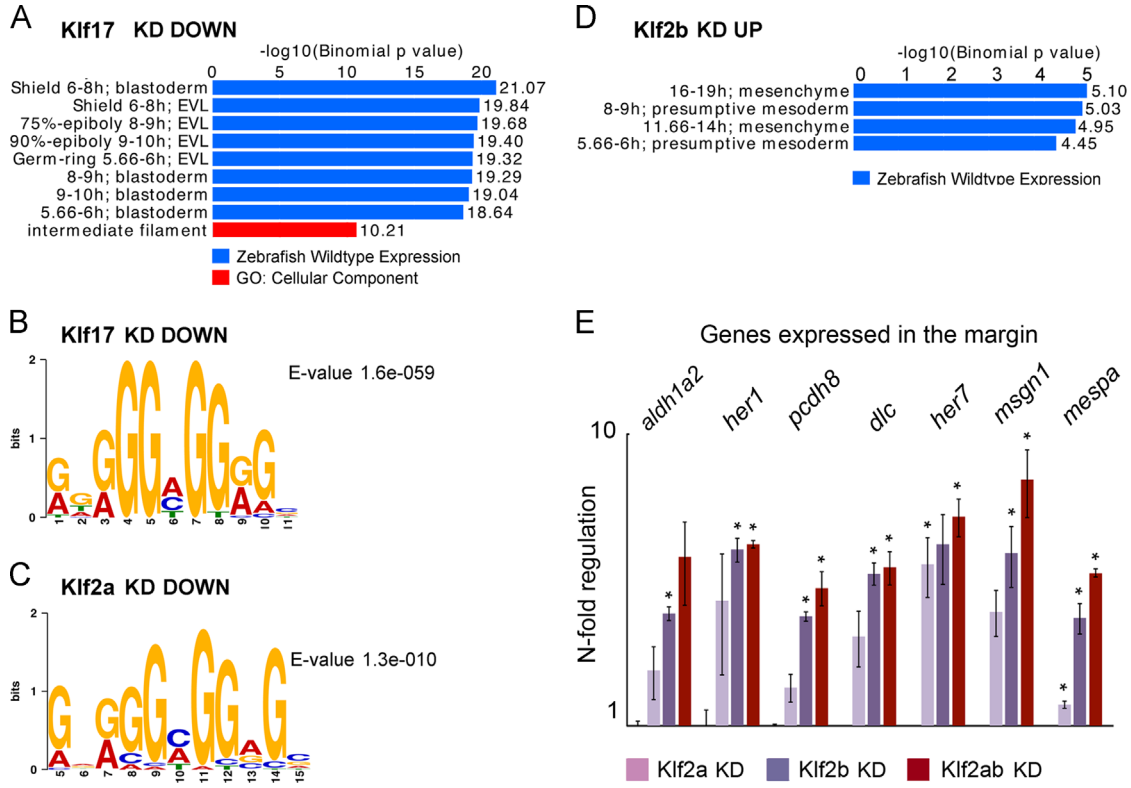

Fig. 5. Reduced expression of EVL-specific genes in Klf17 morphants, but increased expression of mesodermal genes in Klf2b morphants. (A,D) Cellular component and expression pattern annotation of genes downregulated in Klf17 Morpholino KD (A), and genes upregulated in Klf2b KD (D) was performed using GREAT. The charts show the top overrepresented categories belonging to zebrafish expression patterns (blue) and cellular components (red). The *x* axis values correspond to the binomial raw (uncorrected) *P*-values. Genes repressed in Klf17 KD show a statistically significant correlation to genes expressed in the extraembryonic EVL, while genes activated in Klf2b KD show a statistically significant correlation to genes expressed in the mesoderm. (B,C) Position-Weight matrices (PWMs) for the motifs overrepresented in the promoters of genes downregulated after Klf17 KD (B) and Klf2a KD (C) match canonical SP1–Klf4 sites. The *E*-value represents the statistical significance of the motif. (E) Genes normally expressed in the margin are upregulated upon Klf2a, Klf2b individual or double KD. The chart shows fold change values of the microarray probes for *aldh1a2*, *her1*, *pcdh8*, *dlc*, *her7*, *msgn1*, *mespa* in Klf2a KD, Klf2b KD and double KD compared to control Morpholino injected embryos (values for control Morpholino injected embryos are set to 1). Error bars – standard error of the mean, * – *p*-value < 0.05 in Student's *T*-test.

mesoderm anlage at the vegetal margin of gastrula embryos. These seven genes are induced upon Klf2b KD, double Klf2a–Klf2b (Klf2ab) KD, and in two cases (*her7* and *mespa* genes) also following Klf2a KD. We hypothesize that Klf2b and Klf2a may prevent mesodermal genes from being expressed within the Klf2 blastoderm expression domain.

Klfs act downstream of Pou5f1 to activate EVL-specific genes, including epithelial keratins

To identify targets directly activated by Klf17 in the EVL, we analyzed microarray data sets combining three criteria: (1) downregulation in *Klf17* morphants, (2) upregulation upon Klf17 overexpression with CHX treatment, and (3) upregulation upon Klf17 overexpression without CHX treatment (Fig. S7A). Using this approach, we found 5 "direct" target genes: *krt4*, *krt5*, *zgc:92061* (one of two *krt17* genes; (Imboden et al., 1997; Sagerstrom et al., 2005; Pei et al., 2007), *slc14a2* (Hong et al., 2010) and *prph* (Supplementary Table 1). For four of these genes specific expression in the EVL has been described (*krt4*, *krt5*, *zgc:92061* and *slc14a2*). A second set of 43 potentially indirectly regulated target genes are satisfying two of three criteria: they are downregulated in Klf2b morphants, upregulated in Klf17 overexpression without CHX treatment, but not in the presence of CHX. These 43 Klf17 target genes may require zygotic co-factors for activation, or are indirectly regulated through direct Klf17 targets. Some genes in this group, including *capn9*, *sdprb*, zgc:92533 (*krtt1c19a*) and *tagln2*, are also specifically expressed in EVL cells (Pei et al., 2007). The regulation of EVL-specific targets in Klf17 microarray experiments is shown in Fig. 6A. We next confirmed by WISH analysis the EVL expression of *krt4*, *krt5* and *krt17* in WT embryos and the changes in their expression levels in MZ*spg* mutants and Klf17 overexpression as well as KD experiments (Fig. 7). At 6 hpf (shield stage) upon KD of *klf17* we found reduced expression levels of *krt5* (Fig. 7F,G) and *krt17* (Fig. 7K,L), while *Klf17* overexpression in MZ*spg* resulted in ectopic expression of *krt5* and *krt17* (Fig. 7I,J and N,O; arrow). The regulation of *krt4* in MZ*spg* overexpressing *Klf17* observed in microarrays was not detectable by WISH (Fig. 7A, B and D,E).

To identify targets directly repressed by Klf17 in the EVL, we applied the following criteria: (1) upregulation in Klf17 morphants, (2) downregulation upon Klf17 overexpression with CHX treatment, and (3) downregulation upon Klf17 overexpression without CHX treatment (Fig. S7B). This analysis revealed 3 direct and 15 indirect target genes repressed by Klf17 (Fig. S7B, Table S2). None of these 18 genes, except for *klf2a*, are specifically expressed in the EVL.

Next we examined, whether the genes regulated by Klf17 are also regulated by Klf2a and Klf2b. 5 direct and 4 indirect EVL-specific upregulated targets of Klf17 (Supplementary Table 1, Fig. 6A) were also upregulated upon Klf2a and Klf2b overexpression (Fig. 6B,C). Injection of single Klf2a and Klf2b morpholinos into WT embryos had no or little effect on the expression levels of EVL-specific targets (Fig. 6A–C). However, expression of *krt17* was significantly suppressed by Klf17 morpholinos (Fig. 6A), and suppression further

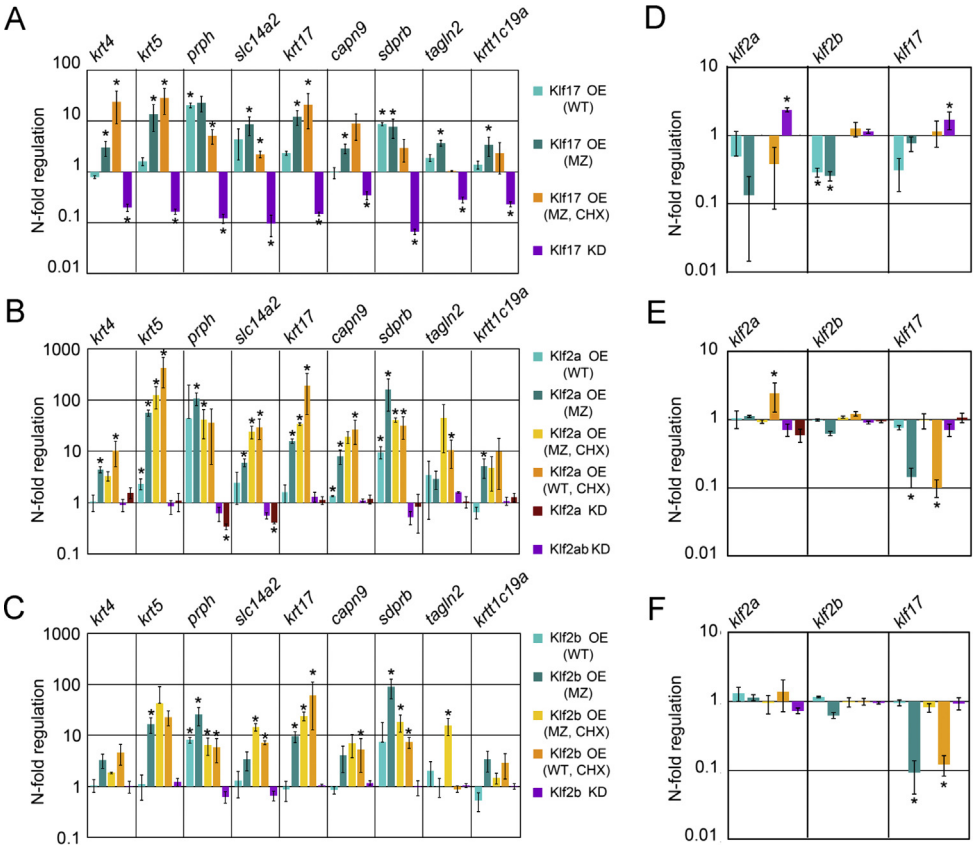

Fig. 6. Klf17, Klf2a and Klf2b induce EVL-specific genes and regulate each other's expression. The charts show fold change values for expression based on microarray probes for 9 selected EVL-specific genes (A–C), or *klf2a*, *klf2b* and *klf17* probes (D–F) in Klf17 (A, D), Klf2a (B, E) and Klf2b (C, F) overexpression (OE) and knockdown (KD) assays compared to control injected embryos. Error bars – standard error of the mean, * – *p*-value < 0.05 in Student's *T*-test. Note, that while EVL-specific genes are induced by all Klfs, they are strongly repressed in Klf17 KD (A), but not in Klf2a or Klf2b KD (B, C). (D–F) Cross-regulatory interaction between *klf17*, *klf2a* and *klf2b* and their protein products. *klf2a* expression level is significantly higher in *Klf17* KD, and *klf2b* expression levels are reduced by Klf17 OE (D). While Klf2a and Klf2b do not significantly affect expression of each other, they directly repress *klf17* in MZ*spg*, but not in WT background (E, F).

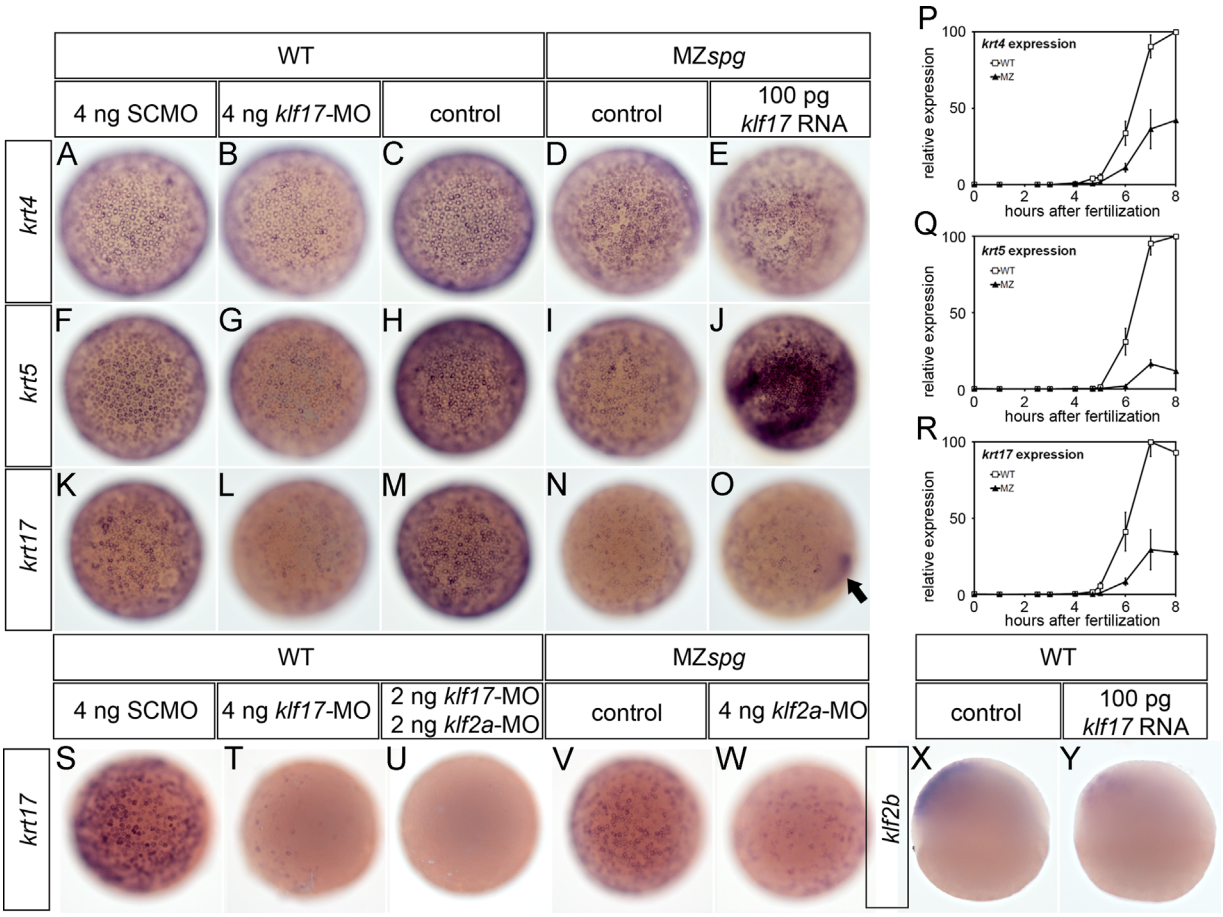

Fig. 7. Klf17 and Klf2a control expression of EVL keratins. (A–O) Expression analysis by whole-mount *in situ* hybridization at 6 hpf, embryos shown in animal view. All three analyzed keratins are expressed in the EVL. The expression of *krt5* and *krt17* are downregulated in Klf17 morphants (F–G and K–L) as well as in Pou5f1 mutant embryos (H–I and M–N). Overexpression of Klf17 in Pou5f1 mutants results in an upregulation of the expression of these two keratins in the EVL and/or to ectopic expression in embryonic tissues (J and O), while expression of *krt4* was only slightly affected (A–E). (P–R) show temporal microarray expression profiles of *krt4*, *krt5* and *krt17* in WT (white squares) and MZ*spg* (black triangles; Onichtchouk et al., 2010). Values are normalized to maximum expression (100). Error bars show SEM of three biological replicates for one probe. (S–W) Whole-mount *in situ* hybridization for *krt17* at 4,7 hpf (30% epiboly), embryos shown in animal view. (S, T, U) Injection of Klf17-MO strongly reduces, and coinjection of Klf2a-MO and Klf17-MO completely suppressed *krt17* expression in WT embryos. (V, W) Klf2a-MO strongly reduces *krt17* expression in MZ*spg*. Whole-mount *in situ* hybridization with *klf2b* probe at 6 hpf, embryos shown in lateral view. (X, Y) Klf17 suppresses expression of *klf2b* in the WT.

enhanced by co-injecting Klf17 and Klf2a morpholinos into WT (Fig. 7S–U). In MZ*spg* embryos, injection of Klf2a morpholino alone could repress most *krt17* expression (Fig. 7V,W).

We next compared the temporal expression profiles of Klf17 targets in WT and MZ*spg* embryos within the first eight hours of development (Fig. 7P–R), using the microarray data from Onichtchouk et al. (2010). Keratins start to be expressed in WT around 30% epiboly, within 1 h after onset of *klf2a* and *klf17* expression. In MZ*spg* mutants the onset of Keratin gene expression is delayed and the expression levels are reduced compared to WT (Fig. 7P–R, compare also Fig. 7C,D, H,I, and M,N). Thus, Pou5f1 supports the specification of the EVL by activating a partially redundant network of Klf17, Klf2a and Klf2b, which in turn control differentiation genes.

Cross-regulatory interactions within the Pou5f1-Klf network in the EVL

We next analyzed cross-regulatory interactions between *klf17*, *klf2a* and *klf2b* using data from our knockdown and overexpression microarray experiments. As shown in Fig. 6D, Klf17 KD induced *klf2a* expression, and Klf17 overexpression reduced *klf2b* levels in the microarray experiment at 30% epiboly in both WT and MZ*spg* (Fig. 6D), and also severely reduced the *klf2b* expression domain at 60% epiboly assayed by WISH (Fig. 7X,Y). Thus, expression of both *klf2b* and *klf2a* in the EVL of WT embryos is negatively regulated by Klf17. Interestingly, Klf2a and Klf2b were able to strongly repress *klf17* only in MZ*spg* mutant embryos, even in the presence of CHX, but not in WT embryos (Fig. 6E,F). One possible explanation for this regulation would be that Klf2a, Klf2b and Pou5f1 directly bind to the same regulatory region of *klf17*. In WT embryos, this regulatory element would be occupied by maternally expressed Pou5f1, which prevents binding and repression by Klf2a and Klf2b. In contrast, in MZ*spg* embryos Klf2a and Klf2b would have access to this regulatory element and mediate repression. To investigate this hypothesis, we mapped the positions of Pou5f1 binding motifs in *Klf17*, *klf2a* and *klf2b* regulatory regions using Pou5f1 ChiP-seq data (Leichsenring, et al., 2013). Direct binding of Pou5f1 was observed in the control and transcribed regions of all three genes (Fig. S8A–C). Next we compared the positions of Pou5f1 binding with the positions matching predicted Klf binding motifs from the promoters repressed by Klf2a (KLF2a OE DOWN (MZ CHX), Fig. S5A) and repressed by Klf17 (Klf17 OE DOWN (MZ), Fig S6). Klf2a and Klf17 binding motifs were found immediately upstream of the transcription start sites of all three *klfs* (Fig. S8A–C), but only in the *Klf17* promoter the Klf2a motif matched the position occupied by Pou5f1. Thus, it is possible that Pou5f1 binding

protects *Klf17* from repression by Klf2a and Klf2b, although further studies are needed to verify this model.

Discussion

Pou5f1/Oct4 is a critical transcriptional regulator of Klf2/4 family genes in early development of both fish and mammals (Ivanova et al., 2006; Onichtchouk et al., 2010). To get insights into the structure and function of zebrafish Pou5f1 and Klf GRNs, we characterized in detail the regulation, spatial expression, and downstream targets of the zebrafish *klf17*, *klf2a* and *klf2b* genes from blastula stage shortly after the onset of the zygotic genome transcription until late gastrula stages. We distinguish three spatial domains of *klf* gene family expression: (I) The EVL domain, shared by all three *klfs*, (Fig. 8A, EVL); (II) a *klf2b* and *klf2a* expression domain in the ventral part of the ectoderm (Fig. 8A, "Ect"); and (III) a *klf17* expression domain in the involuting axial mesoderm giving rise to head axial mesoderm. *klf4a* was not expressed at detectable levels during gastrulation (Fig S9). Comparison of spatial and temporal *klf* expression patterns in wild type as well as MZ*spg* or M*spg* mutant embryos shows that the *klf17* expression domain in axial mesoderm does not depend on Pou5f1. In contrast, proper expression levels of *klf* genes in ectoderm and EVL depend on Pou5f1 activity. While *pou5f1* mRNA has been difficult to detect in the EVL (Takeda et. al., 1994), Pou5f1 protein is present in EVL nuclei during epiboly (Lippok et al., 2013). Our results suggest that the ubiquitously expressed transcription factor Pou5f1 is not the sole activator, but rather strongly potentiates the action of region-specific activators of *klfs* within ectoderm and EVL (Fig. 8B,C). Within the EVL domain Pou5f1 likely cooperates with an EVL-restricted inducer of *klfs*, potentially Irf6, which has previously been invoked in EVL differentiation (Sabel et al., 2009), while in the ectodermal domain Pou5f1 cooperates with BMP signaling to induce *klf2a* and *klf2b*. To address Klf functions within each of the domains during the first hours of zebrafish development, and to resolve the epistatic relationships between Klfs, we analyzed transcriptome changes upon Klf17, Klf2a and Klf2b knockdown and overexpression at late blastula (30% epiboly) stage. We found that within the EVL domain, Klf4 and Klf2 family members together contribute to EVL differentiation, directly inducing EVL-specific genes. Within the ectodermal domain, Klf2b and Klf2a suppress expression of genes characteristic for non-ectodermal fates, specifically mesoderm.

Krüppel-like factors Klf17, Klf2a and Klf2b are redundant inducers of EVL differentiation

Our data suggest that Klf17, Klf2a, and Klf2b are involved in EVL differentiation by activating EVL differentiation genes and presumably blocking the establishment of other embryonic cell fates in the outermost blastula cell layer. Previous studies have shown that Krüppel-like zinc finger transcription factors bind similar promoter sequences to regulate the transcription of their targets (Miller and Bieker, 1993; Bieker and Southwood, 1995; Shields et al., 1996; Shields and Yang, 1998; Yet et al., 1998; Turner and Crossley, 1999; Chen and Bieker, 2001). Zebrafish Klf17, Klf2a, Klf2b, are closely related to Klf2 and we find similar DNA binding motifs, strongly related to previously described Klf4 motifs (Shields and Yang, 1998), enriched in their target gene promoters. Our data suggest that all three Klfs act redundantly in differentiation and development of the extraembryonic EVL by direct binding to the promoter regions of EVL-specific genes.

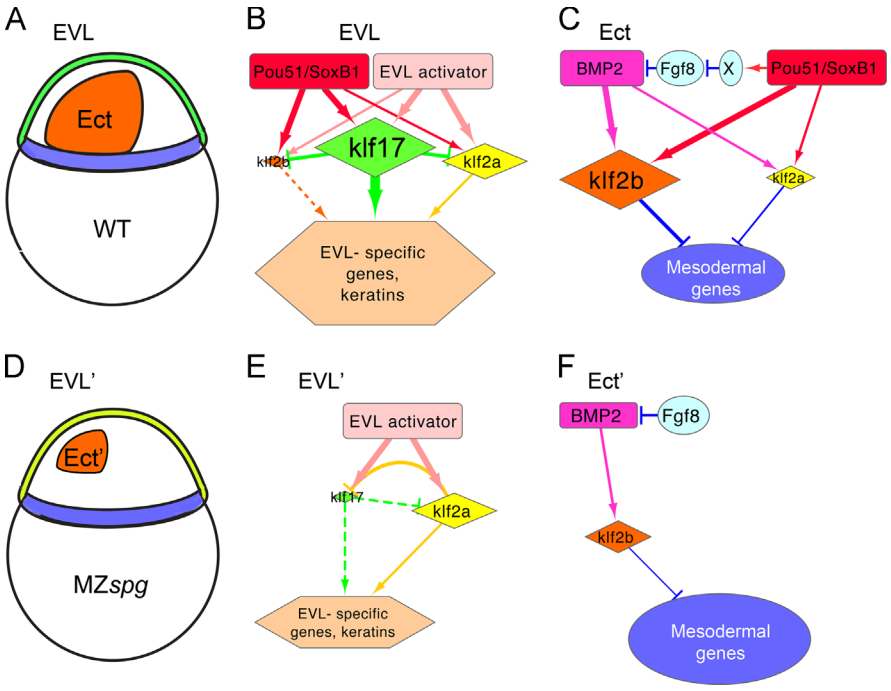

Fig. 8. Model for the Klf17–Klf2a–Klf2b regulatory network in the blastula stage zebrafish embryo. (A) Schematic drawing of zebrafish wild type blastula embryo. *klf17*, *klf2a* and *klf2b* are expressed in the EVL domain (EVL green); *klf2a* and *klf2b* are also expressed in the prospective ectodermal domain (Ect, orange). (B) Klf regulatory network in the EVL domain of WT embryos. *klf17*, *klf2a* and *klf2b* are activated by a tissue-specific EVL activator and by Pou5f1. Klf17 induces EVL-specific genes and represses *klf2a* and *klf2b*. Strength of activation is represented by thickness of arrows. (C) Klf regulatory network in the ectodermal domain of WT embryos. Pou5f1 and BMP signaling (BMP2b) activate *klf2a* and *klf2b*. Klf2a and Klf2b repress expression of mesodermal genes. (D) Schematic drawing of zebrafish MZ*spg* Pou5f1 mutant blastula embryo. *klf2a* and *klf17* are expressed in the EVL domain (EVL', yellow). *klf2b* is expressed in the future ectodermal domain (Ect', orange), which is smaller compared to the *klf2b* expression domain of WT zebrafish. (E) Klf regulatory network in the EVL domain of a MZ*spg* embryo. *klf17*, and *klf2a* are activated to low levels only by a tissue-specific EVL activator. Klf2a induces EVL-specific genes and represses *klf17* expression. Strength of activation is represented by thickness of arrows. Expression of EVL-specific genes (e.g. keratins) is lower than in WT due to reduction in Klf activity. (F) Klf regulatory network in the ectodermal domain of MZ*spg* embryo. BMP signaling (BMP2, reduced in MZspg) activates *klf2b*.

Pou5f1 cooperates with EVL-specific activators to induce Klf genes in EVL

klf17, *klf2b* and, to a smaller extent, *klf2a* expression levels have been reported to be reduced in MZ*spg* mutant embryos (Onichtchouk et al., 2010), but spatial regulation has not been investigated. Whereas the expression of *klf17* and *klf2b* is absent at 30% epiboly in MZ*spg* mutants, *klf2a* expression levels were similar in MZ*spg* mutants and WT. However, *klf2a* expression in the EVL of M*spg* at 30% epiboly was much stronger than both in MZ*spg* and WT, suggesting regulation of *klf2a* by zygotically expressed Pou5f1. Similarly, at 30% epiboly *klf2b* EVL expression in M*spg* was stronger than in WT. The simplest explanation for this pattern is a balanced regulation of *klf2a* and *klf2b* by Pou5f1, by both direct activation and indirect repression via a transcriptional intermediate gene. A good candidate for the Pou5f1-dependent EVL-specific repressor of *klf2a* and *klf2b* is Klf17, a Pou5f1-induced EVL specific gene, expressed to high levels at sphere stage in WT, but not in M*spg* embryos (Fig. 1B,N). Several lines of evidence support that Klf17 is a Pou5f1-dependent repressor of *klf2a* and *klf2b* in the EVL: (i) Klf17 binding sites are present in the promoters of *klf2a* and *klf2b* (Fig S9), (ii) in Klf17 overexpression experiments *klf2a* and *klf2b* are suppressed, while (iii) *klf2a* levels significantly increase upon Klf17 knockdown (Fig. 6D, Fig. 7X,Y). Thus, *klf2a* and *klf2b* are directly activated by Pou5f1 and with *Klf17* form an incoherent feed-forward loop (Macneil and Walhout, 2011), where Pou5f1 directly activates *klf2a* and *klf2b* in the EVL, and at the same time it indirectly represses *klf2a* and *klf2b* through Klf17 (Fig. 8B). This configuration predicts that in the case of Klf17 dysfunction, Klf2a expression will spread to the EVL territory, and take over activation of EVL development. We indeed found this regulation in Klf2a and Klf17 double KD experiments, and in MZ*spg*, where Klf17 activity is severely reduced. Such a functional redundancy of transcription factors is a common principle to achieve higher robustness of biological systems (Masel and Siegal, 2009; Macneil and Walhout, 2011).

Regulation of the EVL specific *klf* expression patterns at blastula stages cannot be explained by Pou5f1 activity alone, because (1) *pou5f1* is uniformly expressed in the pre-gastrulation embryo (Belting et al., 2001; Burgess et al., 2002), and (2) EVL forms normally in MZ*spg*, although its function during gastrulation is not fully normal (Lachnit et al., 2008). Additional factors are needed for the spatial restriction of *klf* expression to EVL. Due to the onset of *klf* expression immediately after MBT, potential factors involved in the spatial regulation are most likely maternally supplied and asymmetrically distributed in the pre-MBT embryo. Therefore one has to postulate an activator located in the EVL and necessary to achieve the full *klf17* and *klf2a* expression levels (Fig. 8B). Several candidate activators of EVL-specific gene expression, including Interferon regulatory factor 6 (Irf6), Inhibitor of NFkB Kinase 1 (IKK1) and Forkhead Box H1 (FoxH1), have been identified (Pei et al., 2007; Sabel et al., 2009; Fukazawa et al., 2010). A recent study (de la Garza et al., 2013) showed that *klf17* and *klf2b* are among the EVL-specific targets whose expression is inhibited by a dominant-negative variant of Irf6 (dnIrf6). Thus, Pou5f1 potentiates the action of an EVL-specific activator, presumably Irf6, to induce Klf genes and ensure robust activation of EVL-specific genes (Fig. 8A,B).

Pou5f1 and BMP signaling act together to induce klf2a and klf2b in the ectodermal domain

By performing gain- and loss-of-function experiments with BMP pathway components, we found that starting from sphere stage the ectodermal expression pattern of *klf2b* is tightly regulated by the BMP pathway. BMP signaling levels are reduced by several mechanisms in MZ*spg* embryos (Belting et al., 2011; Reim and Brand, 2006). In particular, *bmp2b* is indirectly suppressed by excess of Fgf8a at sphere stage in MZ*spg* embryos. However, although inhibition of FGF signaling in MZ*spg* restores *bmp2b* expression (Belting et al., 2011), it did not induce *klf2b* expression in MZ*spg* mutant embryos (Fig. 3C,F). This result suggests that Pou5f1 not only regulates *klf2b* by maintaining BMP signaling levels, but Pou5f1 also directly potentiates effects of BMP on *klf2b* expression.

In comparison to the effects of BMP signaling on *klf2b*, effects of BMP signaling on *klf2a* expression were weaker. Although injection of *bmp2* mRNA induced *klf2a* expression throughout the blastoderm (Fig. 3M,P,S,W), inhibition of FGF signaling did not increase *klf2a* expression at sphere or 30% epiboly stages. At 75% epiboly, where BMP signaling levels in the ventral part of the embryo are much higher than at early gastrula stages (Tucker et al., 2008), the *klf2a* expression domain is “nested” within the *klf2b* expression domain, indicating that *klf2a* may need higher levels of BMP signaling to be induced, when compared to *klf2b*. In 75% MZ*spg*, residual levels of BMP signaling suffice to induce *klf2b*, but not *klf2a*. As Pou5f1 directly binds to the regulatory regions of both *klf2* paralogs (Fig S8), it is well possible that it interacts with downstream components of the BMP pathway in the promoters of *klf2a* and *klf2b*, although direct binding assays would be needed to corroborate this hypothesis. Thus, Pou5f1 potentiates the action of BMP signaling to induce *klf2b* and *klf2a* genes in the developing ectoderm (Fig. 8A,C)

Klfs may act as repressors of alternative cell fates

Cryptic, intra-territorial repression of other possible regulatory states is a widespread design feature of developmental GRNs (Davidson and Levine, 2008; Davidson, 2010). As underlined by Davidson often the function of intra-territorial repressors is to prevent a set of cells from responding to a signal that they are exposed to. Functions of intra-territorial repressors are not revealed until they are experimentally interrupted. Knockdown of Klf2b, or double knockdown of Klf2a and Klf2b function at late blastula led to upregulation of a gene set normally expressed in the mesoderm at later stages of development. This observation suggests that a function of Klf2b (and, presumably, function of Klf2a at later stages) is to keep marginal mesodermal genes silent within the ectodermal domain.

Pou5f1 may promote robustness and evolvability in the downstream GRNs

Changes in the regulatory regions of key developmental genes (cis-regulatory changes), resulting in spatial changes of their expression patterns, are considered to be the main driving force of morphological change in evolution (Carroll, 2008). Frequently occurring regulatory mutations, however, have a negative effect on development or fitness of the organism, if the expression of an important developmental gene is switched off in one of its domains. We suggest here that Pou5f1-activation of three closely related Klf factors is an example for a mechanism which buffers negative consequences of regulatory mutations of individual Klf factors. Indeed, if the EVL expression domain of any single one of the Klf genes is lost as a result of a regulatory mutation in the system shown in Fig. 8B, expression of EVL Keratin family genes would not change (for Klf2a, Klf2b), or would transiently drop and quickly restored in case of Klf17 loss. In the last scenario, Klf17 repression of *klf2a* and *klf2b* will be relieved, and they will be activated by Pou5f1 to higher levels in the same spatial domain. This situation is observed in 30% epiboly M*spg* mutants (Fig. 2E,M), where Pou5f1 is provided, but *klf17* expression is much lower than

in WT (Fig. 1F,N). In EVL of MZ*spg* mutants (Fig. 8E), the sum of Klf activity within the EVL is lower than in WT embryos, resulting in lower activation level of EVL targets and potential reduction of embryo fitness.

Another example of a cross-regulatory network of closely related genes with overlapping expression patterns is the redundant system of the zebrafish ventral repressors *vox*, *vent*, and *ved* acting during blastula and gastrula stages (Imai et al., 2001; Gilardelli et al., 2004). Pou5f1 directly binds to the regulatory regions of all three genes (Leichsenring et al., 2013), and activates *vox* and *vent* with different strength (Belting et al., 2011). The Xenopus genome encodes several functional homologs of *vents*, Xvent1, Xvent2 and their paralogs (Gawantka et al., 1995; Onichtchouk et al., 1996), expressed within overlapping domains and acting redundantly in a ventral specification pathway (Onichtchouk et al., 1998). Cao et al. (Cao et al., 2004) reported that Xvent2B is a directly activated target of Oct25, Xenopus homolog of Pou5f1. It would be interesting to know if other members of the Xvent network are also directly regulated by Xenopus Pou5f1 homologs.

Both the Klf and Vent networks consist of several closely related transcriptional regulators, repressing each other, and sharing the same function. In both cases several members of the network are directly activated by Pou5f1, suggesting that such a regulatory mode may be a more common phenomenon during early development of vertebrates, Along with stabilizing the regulatory output of such a redundant network, Pou5f1 may confer evolvability (Kirschner and Gerhart, 1998) to its components by reducing constraints on expression pattern change of one or several members of the redundant network without compromising its functional output. If this is a widespread phenomenon, one would expect to find higher evolutionary plasticity, in terms of more frequent gene expression pattern changes and functional replacements between related genes, at early developmental stages when Pou5f1 is expressed. Such a high evolutionary plasticity in the development of three mammalian species was indeed documented in a comparative study by Xie et al., (2010).

Materials and methods

Fish and embryo care

We used WT embryos of AB x TL strain crosses (http://www.ZFIN.org) and MZ embryos carrying the *m793* allele of the *spg* mutation (Belting et al., 2001; ZFIN ID: ZDBGENE-980526-485, ZDB-GENO-081023-1). Fish were raised, maintained and crossed under standard conditions as described (Westerfield, 2000). Embryos obtained from crosses were raised in egg water or in 0.3 × Danieau's solution at 28.5 °C. The developmental stage of MZ*spg* embryos was indirectly determined by observation of WT embryos born at the same time and incubated under identical conditions.

Molecular cloning, plasmids, in situ hybridization

Coding regions of *klf4a* (402–823 bp), *klf17*, *klf2a* and *klf2b* were PCR-amplified from gastrula stage zebrafish cDNA using Advantage Taq polymerase (Invitrogen) and following primers: *klf4a*: 5′-CCCAGATATCAGCGACGTTT-3′, 5′-CCTGCGGAAATCCAGAATAA-3′, *klf17*: 5′-GGATCCGCCTCGGACTGAATGAGAGT-3′, 5′-CTCGAGTGC TGTCCCCAAAATAGGAG-3′, *klf2a*: 5′-GGATCCGGAAGGATGAACTG-GACAGG-3′, 5′-CTCGAGGCGTTTAGTCCACATTTTCCA-3′, *klf2b*: 5′-GAATTCCGCACACAATTGGTCTAGGA-3′, 5′-TACGTACATCGTTGTGCA TTTTCCAC-3′. PCR products were cloned to PCR-TOPO2 vector (Invitrogen), sequenced, and subcloned to CS2+ vector for RNA preparation. *bmp2b* and *fgf8a* and *noggin1* expression plasmids were generous gifts from M. Hammerschmidt and B. Thisse. Whole mount in situ hybridization was performed as described in Belting et al. (2011)

Morpholinos

Morpholino oligos Klf17-ATG-MO: 5′-TGCAAATGTTAGGGAACT-CAGAAGG-3′ (Kawahara and Dawid, 2001), Klf2a-ATG-MO: 5′-GGACCTGTCCAGTTCATCCTTCCAC-3′ and Klf2b-ATG-MO: 5′-AGG-CAAGGTAAAGCCATGTCCACGC-3′ were ordered from Gene Tools. To test the specificity of Morpholino oligos, sequences complementary to Klf17-ATG-MO and Klf2b-ATG-MO were cloned upstream of the GFP ORF in the CS2+ vector, to obtain *Klf17*-GFP and *Klf2b*-GFP constructs. 50 pg/embryo mRNA synthesized from *Klf17*-GFP and *Klf2b*-GFP was injected into freshly laid eggs with or without 2–4 ng of the corresponding Morpholino (Fig S6). For microarray analysis of Klf Knockdown experiments, we injected 4 ng/embryo of Klf17, Klf2a or Klf2b morpholino (single KD) or 2 ng/embryo each of Klf2a and Klf2b morpholino (double KD) into 1-cell stage embryos. As control we injected 4 ng/embryo of standard control morpholino (Control-MO, Gene Tools).

Microinjections

mRNAs were synthesized using mMachine kit (Invitrogen) according to manufacturer's instructions. mRNA or morpholinos were injected into the yolk of freshly fertilized eggs (younger than 15 minutes) mounted on 1% (w/v) agarose ramps, using microinjection pipettes connected to an air pressure driven microinjector. A volume of 0.5–1 nl, containing mRNA or morpholino and 0.5% (v/v) phenol red in water, was injected into each zygote.

Microarray-based transcriptome analysis

For the microarray analysis the total RNA of 60–100 embryos per sample, frozen in liquid nitrogen at 30% epiboly (t=4,7 hpf), was isolated using the RNA Easy kit (Qiagen). The Sample quality was assessed using an Agilent Bioanalyzer 2100 with the RNA 6000 nano Assay Kit. Samples were processed by Agilent Technologies Two-Color Microarray-Based Gene Expression Analysis kit, hybridized with Agilent 22 K zebrafish arrays for the Klf17 gain-of-function (Klf17 OE) experiment, and Agilent 44 K zebrafish arrays (GEO identifiers GPL7302 and GPL14664) for the Klf17, Klf2a and Klf2b KD, as well as Klf2a and Klf2b OE experiments. Microarrays were scanned on an Agilent scanner and processed using the GE2-107_95_Sep09_QC Agilent protocol. All experiments were performed in duplicates or triplicates using three independent RNA isolations. Cy3 and Cy5 channels were split in Genedata Expressionist and Analyst software, quantile normalized, and used for independent comparisons. Data were normalized across all experiments and genotypes using quantile normalization (Genedata). Genes were named as regulated for the individual experiment, when they showed a >2-fold difference in signal and a significance of $p<0.05$ (Paired t-test).

Microarray data are deposited in GEO under Superseries number GSE45013, consisting of SubSeries as following: Klf17 OE: GSE44982, Klf2a and Klf2b OE: GSE45010, Klf17 KD: GSE45012, Klf2a and Klf2b KD: GSE45011. Link for the reviewers: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=dxaxlgkyyoeowlc&acc=GSE45013

Cycloheximide experiment

MZ*spg* embryos were microinjected with *klf17*, *klf2a* or *klf2b* mRNA at the 1-cell stage or left non-injected as genetic control.

Embryos were treated with 15 mg/ml of the inhibitor of protein translation cycloheximide (CHX, Calbiochem) dissolved in egg water. CHX was added at 1.5 hpf to allow for translation of injected mRNAs, but to block translation of the earliest zygotic transcripts. In presence of CHX, direct zygotic Klf targets are transcribed, but these mRNAs are not translated, avoiding indirect downstream regulatory effects.

Data analysis and visualization

All sequence analyses of differentially expressed genes was performed in UCSC Galaxy (Goecks et al., 2010); http://main.g2.bx.psu.edu/). For processing of the microarray probes, we used Genedata Analyst (Genedata AG, Basel, Switzerland). To build the interaction schemes of Pou5f1, BMP, Klf2a, Klf2b, and Klf17 (Fig. 8) we used biological network visualization software "Cytoscape" (Shannon et al., 2003).

De-novo motif discovery and motif similarity searches

To determine enriched motifs in the promoters of genes differentially regulated in each of our microarray experiments, we first retrieved the 200 bp sequences upstream of the transcription start site for all these genes from the Zv9 genome assembly, using UCSC Galaxy. Then, the 200 bp upstream sequences for each of the differentially regulated gene sets (test set) were analyzed for commonly occurring motifs using the MEME tool (Bailey and Elkan, 1994), using 300 promoters of randomly taken RefSeq genes as a background set, with the command line: meme < filename > .fasta –neg < 300 control promoters > .fasta -alpha dna -minw 6 -maxw 20. Resulting Position-Weight Matrices (PWMs), which were significantly overrepresented over the background set (*E*-value < 0.01) were selected. Position-specific probability matrix for PWMs shown at Fig. 4B, Fig. 5B,C and Fig. S5A are listed in Table S3. Similarity of these Position-Weight matrices to known motifs from the JASPAR database (Bryne et al., 2008) was quantified by TOMTOM program (Gupta et al., 2007) with following parameters: tomtom -no-ssc -oc. -verbosity 1 -min-overlap 5 -mi 1 -dist pearson -evalue -thresh 10 < filename > .fasta db/JASPAR_CORE_2009.meme db/uniprobe_mouse.meme. All PWMs show similarity to known mammalian SP1 and Klf17 sites (p-value < 0.01). Upstream regions of Klf17, Klf2a and Klf2b were scanned for occurrences of Klf17 OE DOWN (WT) and KLF2a OE DOWN (MZ, CHX) PWMs using the Match program (Biobase, (Kel et al., 2006)).

Enrichment for gene annotations within differentially regulated gene sets

To analyze correlations between Klf2a, Klf2b and Klf17 target gene sets and zebrafish genes grouped by their expression patterns and functions we used the GREAT annotation tool at http://bejerano.stanford.edu/great/public/html/ (McLean et al., 2010). Although GREAT was designed for working with genomic regions, there is a way to use it with gene sets, according to the instructions of developers. First, we mapped gene identifiers from Agilent microarrays to the zebrafish custom gene list used by GREAT (from http://bejerano.stanford.edu/help/display/GREAT/Genes#Genes-SetofGenesforGREAT2.0). Than we retrieved 200 bp sequences upstream of the transcription start site for the sets of genes differentially expressed in microarray experiments, and submitted these lists to GREAT, changing "associated gene rule settings" to "single nearest gene". GREAT than uses annotations from different ontologies to associate genes with annotations, and calculates statistical enrichment for associations between gene sets and annotations, using all genes in the genome as a background.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.10.025.

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